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Regulation of Hexitol Catabolism in *Streptococcus mutans*

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Regulation of hexitol catabolism was investigated in *Streptococcus mutans*, a cariogenic human dental plaque bacterium. Induction of hexitol catabolic enzymes and phosphoenolpyruvate:hexitol phosphotransferase and hexitol phosphate dehydrogenase activities was regulated by an inducer exclusion mechanism initiated by D-glucose and 2-deoxy-D-glucose. Kinetic analysis of the inhibitory effect of 2-deoxy-D-glucose on initial hexitol uptake illustrated that this was a noncompetitive type of inhibition. In mutant strains of *S. mutans* lacking phosphoenolpyruvate:glucose phosphotransferase activity, 2-deoxy-D-glucose was unable to inhibit hexitol uptake. These observations provide evidence for possible molecular mechanisms for the exclusion process.

Glucose, mannitol, and sorbitol are all transported and phosphorylated in *Streptococcus mutans* by a phosphoenolpyruvate (PEP):sugar phosphotransferase (PTS)-mediated mechanism (2, 9, 15). In enteric bacteria and *Staphylococcus aureus*, transport and phosphorylation of PTS substrates occur by the following series of reactions (4, 14, 16):

$$\text{PEP} + \text{enzyme I} \rightleftharpoons \text{pyruvate} + \text{enzyme I}\sim\text{P} \quad (1)$$

$$\text{enzyme I}\sim\text{P} + \text{HPr} \rightleftharpoons \text{enzyme I} + \text{HPr}\sim\text{P} \quad (2)$$

$$\text{HPr}\sim\text{P} + \text{enzyme III} \rightleftharpoons \text{HPr} + \text{enzyme III}\sim\text{P} \quad (3)$$

$$\text{enzyme III}\sim\text{P} + \text{sugar}_{out} \xrightleftharpoons{\text{enzyme II}} \text{enzyme III} + \text{sugar-P}_{in} \quad (4)$$

Enzyme I and HPr (heat-stable protein) are sugar-nonspecific cytoplasmic proteins whereas enzyme III and enzyme II are sugar-specific, membrane-associated proteins. The PTS is not well characterized in *S. mutans*, but the available information suggests that it is similar to those found in enteric bacteria (9; S. S. Dills, unpublished data). Glucose 6-phosphate undergoes enzymatic isomerization to form fructose 6-phosphate whereas hexitol phosphates are dehydrogenated to fructose 6-phosphate by specific pyridine nucleotide-linked dehydrogenases (2). Further dissimilation occurs via glycolysis. Wittenberger and co-workers (2, 9) established that the enzymes unique to hexitol catabolism, mannitol-PTS and mannitol 1-phosphate dehydrogenase for mannitol and sorbitol-PTS and sorbitol 6-phosphate dehydrogenase for sorbitol, were present only in cells grown on mannitol or sorbitol, respectively. They also observed that glucose addition to the medium of a culture growing on either hexitol results in a reduction of hexitol phosphate dehydrogenase activities to levels found in glucose-adapted cells (2). This loss was attributed to catabolite repression by glucose, but a mechanism for this regulatory phenomenon was not suggested.

The results reported herein show that glucose repression of hexitol catabolism is, at least in part, due to inducer exclusion. Evidence for possible molecular mechanisms for the exclusion process is presented.

MATERIALS AND METHODS

Bacteria and culture conditions. *S. mutans* g6715 was obtained from W. J. Loesche, Department of Microbiology, School of Dentistry, University of Michigan, Ann Arbor, Mich. Glucose (enzyme II)-negative mutants were isolated from this parent by selection of 2-deoxy-D-glucose (2DG)-resistant strains, following previously described procedures (18). Cultures were grown anaerobically at 37°C in a complex medium consisting of the following ingredients (per liter): tryptone (Difco Laboratories), 20 g; K_2HPO_4, 2 g; KH_2PO_4, 1 g; NaCl, 2 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $MnCl_2 \cdot 4H_2O$, 0.02 g; and 50 mM carbohydrate unless otherwise indicated in the text.

Enzyme assays. The uptake of ^{14}C-labeled carbohydrates was followed by previously described filtration procedures (14). Rates of PEP-dependent sugar phosphorylation were determined through isolation of the radioactively labeled sugar phosphates by ion-exchange chromatography (7). Hexitol phosphate dehydrogenase activities were determined by following the reduction of NAD spectrophotometrically at 340 nm by previously described methods (2).

Analytical procedures. After cell removal by centrifugation, extracellular (broth) glucose levels were determined by the anthrone assay (5). Protein estima-

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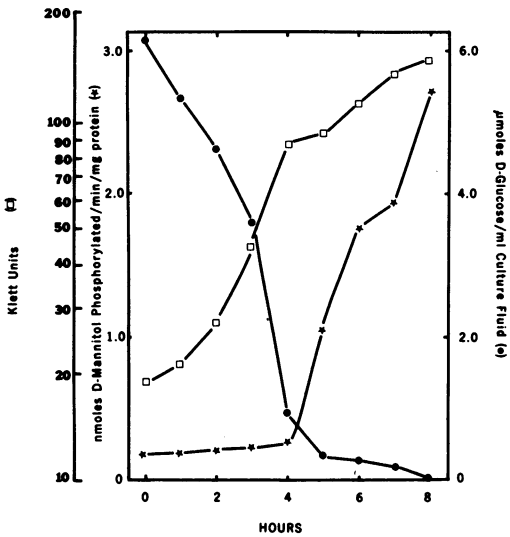

FIG. 1. Catabolite repression of hexitol catabolism in *S. mutans* g6715. Culture conditions were as described in the text. Growth was monitored turbidometrically with a Klett-Summerson colorimeter equipped with a no. 66 (640 to 700 nm) filter. Glucose levels and mannitol-PTS activities were determined as described in the text.

tions were determined by the method of Lowry et al. (8).

RESULTS

Catabolite repression of hexitol catabolism. Brown and Wittenberger (2) observed that when glucose is added to *S. mutans* cells growing on either mannitol or sorbitol, the levels of mannitol 1-phosphate dehydrogenase or sorbitol 6-phosphate dehydrogenase, respectively, decrease to levels found in glucose-adapted cells. We observed that the induction of hexitol catabolic enzymes cannot occur until glucose is depleted from the medium (Fig. 1). Cells were grown overnight on 5 mM glucose, harvested, washed, and then reinoculated into fresh medium containing 5 mM glucose and 5 mM mannitol. Growth was monitored turbidometrically, and samples were withdrawn at the indicated times for determination of extracellular glucose levels and mannitol-PTS activity. No increase in mannitol-PTS activity, indicative of mannitol catabolism, was detected until glucose was exhausted from the medium. Induction did occur once glucose levels fell below 1 μmol/ml, and this corresponded with the lag in the biphasic growth curve. When cells were grown overnight on glucose and then inoculated into fresh media containing mannitol as the sole carbohydrate, no lag in growth occurred (data not shown).

Induction of sorbitol-PTS activity followed a similar pattern. However, growth on sorbitol proceeded only after a considerable lag period (2 to 3 h). It was, therefore, difficult to observe a direct correlation between glucose depletion from the medium and induction of sorbitol-PTS activity. Addition of glucose to cells growing on sorbitol did result in the reduction of sorbitol-PTS activity to uninduced levels (data not shown).

Inducer exclusion of hexitol uptake. The data presented in Fig. 1 confirm the earlier observation (2) that glucose represses the induction of hexitol catabolic enzymes. A similar phenomenon exists in *Escherichia coli* in which glucose represses the synthesis of β-galactoside catabolic enzymes. The molecular mechanism involved in this repression process is in part due to inducer exclusion, that is, induction of the β-galactoside catabolic enzymes is prevented by inhibition of inducer transport into the cell by glucose (4). The data presented in Fig. 2 show that a similar condition exists in *S. mutans*. Both glucose and 2DG caused an immediate inhibition of mannitol and sorbitol initial uptake by whole cells. The inhibitory capacity of both hexoses was approximately equal, and since 2DG is taken up and phosphorylated by the *S. mutans* glucose-PTS but not further metabolized (15), it is probable that this inhibition is due to glucose rather than to a buildup of a metabolic intermediate. In addition to glucose and 2DG, fructose and sucrose also elicited hexitol exclusion in *S. mutans* (data not presented).

Both sugar transport and phosphorylation are catalyzed concomitantly by the PTS (4). It was, therefore, expected that glucose and 2DG would also inhibit PEP-dependent hexitol phosphorylation. As is shown in Table 1, this was found to be true. Rates of hexitol phosphorylation with cell extracts prepared from either mannitol- or sorbitol-grown cells were determined in the absence (control) or presence of glucose, 2DG, or glucose 6-phosphate. Both glucose and 2DG caused severe inhibition of hexitol phosphorylation whereas glucose 6-phosphate, the immediate product of the glucose-PTS, caused only slight inhibition. These results support our earlier hypothesis that the free hexoses are the actual inhibitors of hexitol uptake rather than a metabolic intermediate.

The other possible regulatory sites of enzymatic activity unique to hexitol catabolism are the hexitol-phosphate dehydrogenses. The activities of these enzymes assayed in the same cell extracts described above were not inhibited by glucose, 2DG, or glucose 6-phosphate (Table 1).

Molecular mechanism for inducer exclusion process. Two experimental approaches have given evidence for a possible molecular mechanism for glucose-mediated exclusion of hexitols.

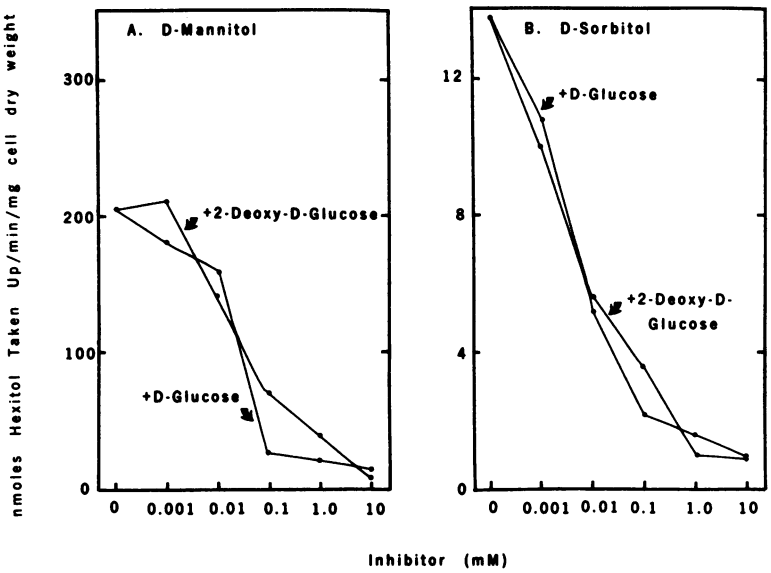

FIG. 2. Inhibition of initial hexitol uptake by D-glucose and 2DG in *S. mutans* g6715. Uptake assays of ^{14}C-labeled mannitol or sorbitol were conducted as previously described (14). Inhibitors (D-glucose or 2DG) were added simultaneously with hexitol addition. Initial hexitol concentration, 100 μM; inhibitor concentration, 1 mM.

These are kinetic analyses of the inhibition of hexitol uptake by hexoses and isolation and characterization of mutants of *S. mutans* g6715 that are deficient in glucose-mediated regulation of hexitol uptake.

Kinetic analysis of the inhibitory effect of 2DG on initial mannitol and sorbitol uptake illustrated that inhibition occurred noncompetitively (Fig. 3). Affinity (K_m) values remained constant at micromolar levels whereas maximum velocity values decreased with increasing 2DG concentrations. Glucose had the same inhibitory effect as 2DG (data not shown). It is clear that 2DG and glucose do not compete with hexitols for the same site on a common transport protein.

TABLE 1. Regulation of hexitol catabolic enzyme activities in cell extracts of *S. mutans* g6715

Inhibitor[a]	Substrate[b]			
	Mannitol-PTS	Sorbitol-PTS	Mannitol 1-phosphate dehydrogenase	Sorbitol 6-phosphate dehydrogenase
None	1.3	13.4	43.1	28.2
Glucose	0.3	3.4	40.6	31.2
2DG	0.2	4.6	48.4	26.1
Glucose	1.1	ND[c]	38.2	25.3

[a] Final concentrations, 1 mM.

[b] Enzyme assays were conducted with cell extracts prepared by published procedures (3) from cells grown in the presence of either mannitol or sorbitol. PTS activities are given as nanomoles of hexitol phosphorylated per minute per milligram of protein. Dehydrogenase activities are given as nanomoles of NAD reduced per minute per milligram of protein.

[c] ND, Not determined.

Spontaneous mutants of *S. mutans* g6715 incapable of growth on D-glucose were isolated by screening for 2DG resistance in the presence of mannitol or sorbitol as growth substrates. It was presumed that a mutation conferring 2DG resistance as well as allowing growth on mannitol and sorbitol would have to lie in either a regulatory or structural gene coding for the enzyme II specific for glucose (enzyme II^{glc}). This selection procedure is based on the observations that in *S. mutans* both hexitols and glucose (and 2DG) are transported into the cell by a PTS-mediated mechanism (9, 15). Both substrates would utilize the same pool of sugar-nonspecific PTS proteins (enzyme I and HPr) for transfer of the phosphate from PEP to a sugar-specific integral membrane protein (enzyme II). Evidence has been reported demonstrating that *S. mutans* possesses separate sugar-specific enzyme IIs for glucose, mannitol, and sorbitol (9). Of the approximately 30 mutant strains isolated by 2DG resistance selection, all had a similar pattern of PTS activities, as is shown for two mutant strains in Table 2.

Both strains 2DGr1 and 2DGr11 possessed 50- to 100-fold lower glucose- and 2DG-PTS phosphorylation rates than did the parent strain. However, mannitol-PTS activities were at comparable levels in all three strains. These data indicate that the mutational defect resulting in loss of glucose phosphorylation activity resides

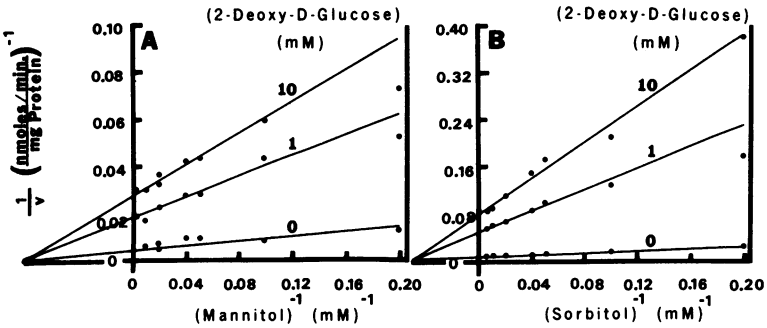

FIG. 3. Lineweaver-Burk plots showing the effect of various 2DG concentrations on initial D-mannitol (A) and D-sorbitol (B) uptake by *S. mutans* g6715. Uptake assays were conducted as described in the legend to Fig. 2. Initial hexitol concentration, 100 μM.

within the gene coding for enzyme II^{glc}. If the defect was in one of the genes coding for the nonspecific PTS proteins (enzyme I or HPr), then a loss of mannitol-PTS activity corresponding to the loss of glucose-PTS activity should have been detected.

Our previous data suggested that glucose inhibited hexitol uptake through direct interaction of the glucose transport system with the hexitol transport systems. Therefore, inducer exclusion of hexitol uptake should not be observed in mutants lacking the enzyme II^{glc}. Glucose-mediated regulation was either greatly reduced or completely absent in the mutant strains 2DGr1 and 2DGr11 (Table 3). These data support the hypothesis that the glucose-transporting mechanism interacts directly with the hexitol transport system, thereby inhibiting hexitol uptake.

Carbohydrate-stimulated lactate efflux from *S. mutans*. Both glucose and 2DG caused a severe inhibition of initial hexitol uptake when the inhibitor and substrate were added simultaneously (Fig. 2). It was also expected that in an experiment in which hexitol uptake by whole cells was followed over an extended period, 2DG addition would cause an immediate cessation of hexitol accumulation. This did occur; however, 2DG also stimulated a release of radioactive material from the cells (Fig. 4). Similar results were obtained for the effect of 2DG on sorbitol uptake. Furthermore, fructose, glucose, 2DG, sucrose, and additional mannitol stimulated radioisotope efflux from cells provided with labeled mannitol; water and lactose did not (data not presented). All of these substrates, except lactose, are recognized and accumulated by mannitol-grown cells. The system for lactose uptake must be induced by growth on lactose. These observations show that the stimulation of the radioisotope efflux is dependent upon cell recognition of a transportable carbohydrate. Metabolism of the efflux inducer past phosphorylation is not necessary since 2DG also elicits the response.

Differentiation of exclusion and expulsion phenomena. Observation of the efflux phenomenon led us to wonder if the inducer exclusion process described above was the operable mechanism for hexitol exclusion or if in fact rapid inducer

TABLE 2. PTS activities in mannitol-grown 2DG-resistant mutants of *S. mutans* g6715

Strain[a]	Substrate[b]		
	Glucose	2DG	Mannitol
Parent	7.6	0.85	3.8
2DGr1	0.2	0.01	8.4
2DGr11	0.04	0.004	1.8

[a] Assays were conducted with toluene-decryptified whole cells by previously described procedures (7, 13).

[b] Results are presented as nanomoles of substrate phosphorylated per minute per milligram of protein.

TABLE 3. Inhibition of initial mannitol uptake by 2DG in 2DG-resistant mutants

Strain	2DG (mM)[a]	Sp act[b]	% Inhibition
Parent	0	70	
	1	25.9	63
	10	29.7	58
2DGr1	0	29.4	
	1	33.4	0
	10	24.0	18
2DGr11	0	86.1	
	1	86.1	0
	10	89.1	0

[a] 2DG was added simultaneously with substrate addition.

[b] Units: nanomoles to mannitol taken up per minute per milligram of protein.

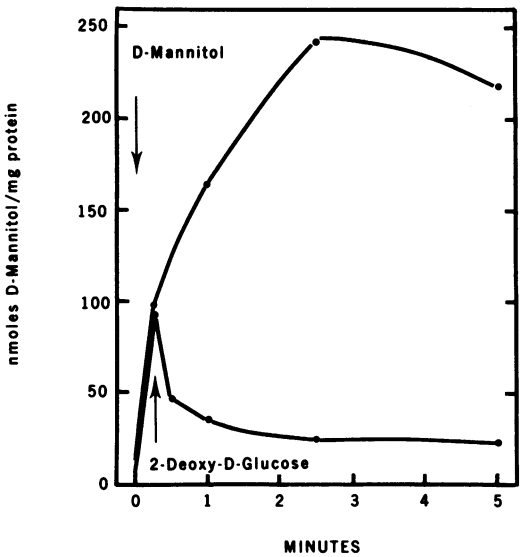

FIG. 4. Uptake of D-mannitol versus time (upper curve) and effect of 2DG (lower curve). Uptake assays were conducted as previously described (14), except that samples were withdrawn from a common reaction mixture at the indicated times. 2DG was added at 15 s after mannitol addition to a 1 mM initial concentration.

expulsion could explain our previous results. Glucose-initiated expulsion of β-galactosides has been previously observed in *Streptococcus pyogenes* and *Streptococcus lactis* (12, 18). The chemical identity of the expelled material was determined by paper chromatography to determine if inducer expulsion was occurring.

2DG-stimulated expelled material was collected from cells given labeled hexitol by filtration through Millipore filters (0.45-μm pore size). The filtrate was concentrated by lyophilization and spotted on Whatman 3MM chromatography paper, and the chromatogram was developed in a descending manner with ethylacetate-pyridine-water (60:25:15). Spots were then developed with either anthrone reagent (reducing sugars) or ammoniacal silver nitrate (carboxylic acids), and chemical identities were determined by comparison of R_f values with those of known standards. Two radioactive spots were separated from the expelled material. Over 95% of the applied counts appeared in a spot that corresponded with a lactic acid standard. The remainder of the labeled material had the same R_f value as pyruvic acid (data not shown). Other metabolic end products known to be produced from hexitols (formic acid and ethanol [1]) were not found in the expelled material. These end products were probably lost during lyophilization.

This observation eliminated inducer expulsion as a mechanism by which the glucose-PTS mediates hexitol acquisition and utilization. It does demonstrate that carbohydrates recognized by *S. mutans* (possibly by the presence of a functioning transport system) stimulate the efflux of metabolic end products from the cell. Otto et al. (10) have demonstrated in *Streptococcus cremoris* the coupled efflux of hydrogen and lactate ions resulting in the formation of a proton motive force (PMF). *S. mutans* may also generate a PMF in this manner.

DISCUSSION

The results presented in this report have demonstrated that inducer exclusion is, at least in part, responsible for the catabolite repression of hexitol metabolism in *S. mutans* g6715 (2; Fig. 1). Several observations have provided evidence for a possible molecular mechanism for this exclusion process. Hexitol exclusion is probably not due to the production of an inhibitory metabolic intermediate since 2DG, a glucose analog taken up and phosphorylated by the glucose-PTS but not further metabolized, inhibited hexitol accumulation (Fig. 2). Intracellular sugar phosphates have been shown to regulate sugar uptake (4). However, it is also doubtful that this type of regulatory mechanism is operable in hexitol exclusion from *S. mutans* since glucose 6-phosphate, the immediate product of the glucose-PTS, did not appreciably reduce PEP-dependent hexitol phosphorylation by cell extracts (Table 1). Kinetic analyses of the inhibitory effect of 2DG on initial hexitol uptake demonstrated that 2DG inhibited in a noncompetitive manner (Fig. 3). Therefore, 2DG (and glucose) were not competing with either mannitol or sorbitol for the same site on a common transport protein. However, the necessity of a functional glucose transport system for regulation was demonstrated by the lack of regulation in mutants deficient in glucose-PTS activity. These results suggest a direct interaction between the glucose and hexitol transport systems.

It is known that glucose, mannitol, and sorbitol have distinct enzyme IIs in *S. mutans* and that the PTS activities for mannitol and sorbitol are fully inducible by growth on mannitol and sorbitol, respectively. As shown in reaction 4 above, enzyme IIs catalyze concomitant sugar translocation and phosphorylation and HPr~P serves as the immediate energy and phosphate source common to all PTS-mediated transport (reaction 3). Competition for HPr~P by the various enzyme IIs has been proposed as a possible mechanism for regulating PTS substrate uptake (4, 17). In this regulatory scheme, substrates whose enzyme IIs have high affinities for HPr~P would be transported in preference to substrates with lower enzyme II-HPr~P affinities.

Other regulatory schemes for glucose-PTS-mediated control of hexitol uptake are certainly possible. For example, the glucose-PTS in *E. coli* is inhibited by a PMF (6, 11). Most of the observations described in this report could be explained by a regulatory scheme in which a high PMF would be required for hexitol uptake but not for glucose transport. If glucose uptake decreased the PMF, then hexitol uptake would be inhibited. Additional work needed to identify the molecular mechanism controlling hexitol uptake is now being undertaken. This includes isolation of mutants with reduced HPr levels in which hexitol uptake should be hypersensitive to glucose-PTS-mediated exclusion and use of proton uncouplers to determine the possible role of a PMF in regulation of hexitol uptake.

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